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Opposite effects of low and high doses of arginine on glutamate-induced nitric oxide formation in rat substantia nigra

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Abstract

L-arginine is a very versatile amino acid that is involved in many important physiological processes such as protein, nitric oxide (NO), agmatine, putrescine, urea, L-ornithine or creatine synthesis and is essential for posttranslational arginylation of protein. The present study was designed to evaluate in vivo the effect of L-arginine on NO production in substantia nigra. In vivo spectroscopic and voltammetric studies were addressed in rats to record modifications in methemoglobin and NO levels under glutamate stimulation. Results showed that, under physiological L-arginine extracellular concentration, the intranigral infusion of glutamate produced an increase in NO levels. When a low dose of L-arginine was co-infused with glutamate, a persistent and higher increase in NO levels was observed. The co-infusion of glutamate with a moderate dose of L-arginine induced drastic and persistent NO production. It was also observed that high doses of either L-arginine or D-arginine inhibit NO production. Subsequently, these data show that L-arginine and D-arginine are involved in a mechanism that inhibits NO production.

Author Keywords: Nitric oxide; Glutamate; Methemoglobin; L-arginine; D-arginine

Article Outline

Nitric oxide (NO) is a highly reactive and multifunctional molecule that can be synthesized in a number of tissues including the brain, where NO is an important intercellular messenger and an intermediary in the actions of excitatory amino acids. Vascular endothelial cells and neuronal tissues contain constitutively expressed NO synthase isoforms that are Ca^{2+}/calmodulin-dependent and generate NO and L-citrulline from L-arginine. Excitatory amino acids increase cytoplasmatic free calcium concentration and activate constitutive NO synthase [7]. In the central nervous system, L-arginine, besides being a precursor of NO and protein synthesis, serves as a precursor of other molecules such as urea, L-ornithine [14], agmatine, putrescine, the precursor of polyamine synthesis [8, 11 and 17] or creatine [1 and 4] and is essential for posttranslational arginylation of protein [10]. In addition, L-arginine may be involved in other physiological processes such as the modulation of cerebral mitochondrial L-glutamate uptake [3]. Previous in vitro studies have shown that some of these pathways may be inter-regulated. For example, agmatine inhibits all isoforms of NO synthase [6] and arginase II activity inhibits NO production [3]. These observations raise questions concerning the interplay between L-arginine and generation of NO. In this study, we examine in vivo the effects of local infusion of three doses of L-arginine on glutamate-induced NO formation in substantia nigra by photometric and voltammetric recordings.

All experiments were carried out on male Sprague–Dawley rats (280–350 g). Animals were housed under a 12-h light/12-h dark cycle with free access to food and water. The compounds used for microinjections were L-glutamic acid (RBI, Natick, MA, USA), L-arginine (Sigma, St. Louis, MO, USA) and D-arginine (Fluka, Buchs, Switzerland). All drugs were dissolved immediately before administration in a phosphate buffer saline (PBS 0.01 M; pH=7.4). Under urethane anesthesia (1.3 g/kg, i.p.), animals were placed in a Kopf stereotaxic frame for photometric or voltammetric recordings from substantia nigra (5.3 mm posterior and 1.8 mm lateral from bregma, and 7.1 mm ventral from the brain surface; incisor bars: −3.4 mm).

Methemoglobin levels were recorded in vivo by optical fibers placed into substantia nigra. Under physiological conditions, NO reacts with oxyhemoglobin to form methemoglobin in a stoichiometric manner ($HbO_2+NO \rightarrow MetHb+NO_3^-$).This reaction has a very high rate, even under saturating oxygen concentrations, and it has been estimated to be at least 26 times faster than the auto-oxidation of NO in aqueous solution. Thus, methemoglobin levels are proportional to NO concentration and they can be used as indirect index of NO [5]. As previously described [2], two quartz optical fibers (100 μm. external diameter) were cemented in parallel and mounted in a carrier assembly. An attached polymicro tubing (Polymicro Tech, England, 100 μm external diameter) allowed the local infusion of drugs (50 μm far from the fiber tip). For spectroscopic measurements in the range of 200–800 nm, light from a 75 W halogen lamp was passed through an optical fiber implanted in substantia nigra. The light scattered by the cerebral tissue was collected by the second optical fiber and sent to a linear CCD detector device (Ocean optics, Eerbeek, Netherlands) via a compact built-in monochromator. The spectrometer and data acquisition were controlled by a personal computer, allowing one to obtain optical absorption spectra (10/s) and temporal evolution of the absorption in different wavelength. The methemoglobin level, expressed in absorbance arbitrary units, was calculated as follows: $\Delta c_{metHb}=\Delta Ab_{632nm-0.56\times 614\ nm-0.44\times 655\ nm}$. The methemoglobin levels were averaged every 5 min to facilitate the analysis. After a stable 10 min period, drugs (0.4 μl) were infused and methemoglobin levels were recorded for at least an additional 20 min period.

The electrochemical procedure used is based on the catalytic oxidation of NO on polymeric metalloporphyrins [12]. Differential double pulsed voltammetry (DDPV) with carbon fiber microelectrodes covered with a polymeric porphyrin film and coated with a perfluorinated polyacid with cation exchange properties (Nafion) was performed as previously described [13]. The working electrode consisted of a carbon fiber (12 μm in diameter, 500 μm in

length) which was covered by electro-deposition, using differential pulse voltammetry (DPV), with a polymeric film of tetrakis (3-metboxy-4-hydroxyphenyl) porphyrin containing nickel as the core metal (Interchim, Moutlucon, France) and successive dippings (total time 15 s) into a 5% solution of Nafion (Aldrich Chemical Co., Milwaukee, WI, USA). These electrode coatings are intended, respectively, to enhance the NO signal and to exclude interfering anions such as nitrite. Thus, these electrodes can detect tissue levels of NO in the nanomolar range [12 and 13]. An attached polymicro tubing allowed the infusion of drugs in the vicinity of the working electrode. A standard three-electrode potentiostat circuit, as commonly used for in vivo voltammetry recordings was completed with a reference electrode (Ag-AgCl) and a counter (or auxiliary) electrode made of stainless steel wire, which were attached to the skull surface and kept wet with saline-soaked pads. Voltammetric recordings were made with a microprocessor controlled potentiostat system (Bioelectrochemical Analyser, ULL, Tenerife, Spain). The following DDPV parameters were used: potential range, −200 to 800 mV; scan rate, 10 mV/s; pulse 1: 40 mV × 40 ms; pulse 2: 80 mV × 40 ms; prepulse duration, 50–120 ms. In these conditions, NO solutions showed an oxidation peak at approximately 650 mV.

At the end of the experiments, animals were deeply anaesthetized and perfused transcardially with heparinized PBS (pH=7.4) followed by 4% (w/v) paraformaldehyde in PBS. The brains were removed, postfixated and cryoprotected. Mesencephalic coronal sections (40 μm) were cut and stained with Cresyl violet and location of voltammetric electrode or photometric sensors were verified.

All results are expressed as mean±SEM. Statistical significance ($P<0.05$) was evaluated by ANOVA and unpaired Student's *t*-test.

In agreement with previous observations that have reported that excitatory amino acids stimulate NO synthesis [7], the injection of 40 nmol of glutamate ($n=8$) significantly increased methemoglobin concentration compared with buffer-treated controls ($n=6$) for about 10 min after intranigral injection (Fig. 1B). The co-infusion of 8 ($n=8$) and 32 nmol ($n=7$) of L-arginine caused a persistent elevation of glutamate-stimulated increases of methemoglobin levels which were statistically significant for at least 20 min after injection (Fig. 1B). As shown in Fig. 1C, which represents the value obtained in the 5–10 min postinjection interval, this latter action of 8 and 32 nmol of L-arginine followed a dose-dependent manner. Thus, 8 nmol of L-arginine enhanced glutamate-induced increases of methemoglobin levels ($P=0.013$) but this action was significantly lower than that caused by 32 nmol of L-arginine ($P=0.039$). In contrast, the co-infusion of 128 nmol of L-arginine with glutamate ($n=7$) had no significant effect on glutamate-stimulated increases of methemoglobin concentration (when compared with buffer-treated controls which only reached significance in the first postinjection five minutes; $P=0.049$; Fig. 1B). In addition, this effect of 128 nmol of L-arginine was lower than that observed with 32 nmol of L-arginine for all the postinjection time intervals ($P<0.05$). The same amount of D-arginine (128 nmol) was able to inhibit glutamate-stimulated increases in methemoglobin concentrations (it was not significantly different from buffer-treated controls in any postinjection 5 min-intervals; and was significantly lower compared with glutamate alone in the two 5 min-intervals in which glutamate enhanced the methemoglobin level: $P=0.037$ and $P=0.026$ in 0–5 min and 5–10 min postinjection, respectively). To further study the inhibition of D-arginine on methemoglobin concentration, in four animals, 40 nmol of glutamate was injected 150 min after the infusion of glutamate plus 128 nmol of D-arginine. As shown in Fig. 1C, which shows a representative example, glutamate plus 128 nmol of D-arginine failed to increase the methemoglobin concentration, but, later, when only glutamate was injected, increases in methemoglobin levels were observed.

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Fig. 1. Effect of arginine on glutamate-stimulated increases in the methemoglobin level in

substantia nigra. Methemoglobin levels were determined by in vivo spectroscopy (AAU: absorbance arbitrary units, glu: L-glutamate, arg: arginine). Drugs dissolved in 0.4 μl (PBS; pH=7.4) were infused into rat substantia nigra (A) Values were averaged every 5 min for each rat and represent mean±SEM (six to eight rats per group).*P<0.05 and #P<0.01 compared with buffer-treated controls in the same time interval. The inset shows an example of spectra recorded from substantia nigra. The arrow indicates the methemoglobin band. The effect of 128 nmol of L-arginine on glutamate-stimulated increases of methemoglobin was lower than that observed with 32 nmol of L-arginine in all postinjection time intervals (P from 0.017 to 0.035) and was not significantly different from glutamate alone. D-arginine (128 nmol) completely inhibited the action of glutamate on methemoglobin levels. Results of 0, 8, 32 and 128 nmol of L-arginine plus 40 nmol of glutamate in 5–10 min postinjection interval are also represented in B. (B) Values, represented by mean±SEM, were obtained for each rat by averaging the recording from postinjection min 5 to 10. *Significantly different (P<0.05) from glutamate alone (0 nmol of L-arginine). #P<0.05 compared with the rest of the groups. (C) A high dose of D-arginine inhibited glutamate-stimulated increases in methemoglobin levels in substantia nigra. Values represent increments related to pre-drug baseline in absorbance arbitrary units in methemoglobin band. Arrows indicate intranigral injection of drugs (0.4 μl). Forty nmol of L-glutamate plus 128 nmol of D-arginine reduced methemoglobin level as vehicle solution (see panel A). However, after 150 min recovery period, 40 nmol of glutamate increased methemoglobin concentration. Similar results were obtained from three additional rats.

In order to further ensure the inhibitory effects of high doses of L- and D-arginine on glutamate-stimulated NO production, direct measures of NO were recorded by voltammetry. As shown in Fig. 2, the injection of 40 nmol of L-glutamate (n=5) enhanced NO production compared with buffer-treated controls (n=5) (P=0.015 and P=0.016 for 10–15 and 15–20 min postinjection intervals, respectively). Glutamate-stimulated NO production was markedly increased by 32 nmol of L-arginine (n=5) in all postinjection time intervals. But, in accordance with methemoglobin results, 128 nmol of L-arginine (n=6) had no significant effect on glutamate-evoked NO production as the NO levels were significantly lower than the corresponding value obtained with 32 nmol L-arginine. Finally, 128 nmol of D-arginine (n=5) inhibited glutamate-induced increases of NO concentration (all time intervals were not significantly different from buffer treated controls).

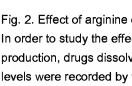

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Fig. 2. Effect of arginine on glutamate-stimulated increases in NO production in substantia nigra. In order to study the effect of high doses of arginine on glutamate-stimulated increases in NO production, drugs dissolved in 0.4 μl (PBS; pH=7.4) were infused into rat substantia nigra and NO levels were recorded by voltammetry techniques. The inset shows an example of pre-drug voltammograms recorded from substantia nigra. The figure shows percentage of pre-drug baseline at the height of NO peak averaged every 5 min. Values are mean±SEM (five to six rats per group). *P<0.05 compared with any group; #P<0.05 when compared with buffer-treated controls.

The results presented here show that, under physiological L-arginine concentration, glutamate produced an increase in NO levels. When the local extracellular levels of L-arginine were enhanced by the co-infusion of L-arginine, dose-dependent increases in glutamate-stimulated NO production were observed. However, when the extracellular concentration of L-arginine was maximal (128 nmol), a drastic reduction of NO production was recorded. These effects can be observed by voltammetric measures of NO and by spectroscopic recordings of methemoglobin. Thus, this in vivo study suggests that L-arginine, besides being a precursor of NO synthesis, is

involved in at least a mechanism that can inhibit NO production. Previous in vitro works have shown that arginase II activity and agmatine can reduce NO synthesis. Given that the reduction of NO synthesis by arginase II activity appears to be a consequence of the depletion of L-arginine [9], it is unlikely that the inhibitory effect reported here was mediated by arginase II because this action was observed only when highest doses of L-arginine were infused. However, it is possible that L-arginine inhibits NO generation by its enzymatic decarboxilation to agmatine. Several studies have shown that agmatine inhibits all isoforms of NO synthase [8] and antagonizes *N*-methyl-D-aspartate receptor [15 and 19]. Finally, D-arginine also caused a strong inhibition on glutamate-induced nitric oxide formation. This suggests that the mechanism by which L-arginine inhibits NO synthesis is not stereoselective. However, a specific action of D-arginine cannot be discarded since previous studies have shown that this D-amino acid can reduce mitochondrial free calcium concentration, depolarizate mitochondria, modify urea biosynthesis and citrulline formation, and reduce mitochondrial ornithine uptake [16 and 18], effects related to the metabolism of L-arginine.

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